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(54) Title: ADULT HUMAN DENTAL PULP STEM CELLS *IN VITRO* AND *IN VIVO*

(57) Abstract: The present invention provides a culture of isolated adult human dental pulp stem cells that can differentiate into dentin/pulp tissue that can be used to produce a tooth in a human being. The present invention further provides a method of regenerating human dentin/pulp tissue.

ADULT HUMAN DENTAL PULP STEM CELLS *IN VITRO* AND *IN VIVO*

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates to the field of restorative dentistry in humans. Specifically, the present invention relates to a population of cells that can differentiate into dentin/pulp and a method of regenerating dentin/pulp.

BACKGROUND ART

During tooth formation, interactions between epithelial and dental papilla cells promote tooth morphogenesis by stimulating a subpopulation of mesenchymal cells to differentiate into odontoblasts, which in turn form primary dentin. Morphologically, odontoblasts are columnar, polarized cells with eccentric nuclei and long cellular processes aligned at the outer edges of dentin (1). Following tooth eruption, secondary dentin is formed by odontoblasts in response to general mechanical erosion or disruption, and through dentinal degradation caused by bacteria (2). These odontoblasts are thought to arise from the proliferation and differentiation of a precursor population, residing somewhere within the pulp tissue (3). Despite extensive knowledge of tooth development, and of the various specialized tooth-associated cell types, little is known about the characteristics and properties of their respective precursor cell populations in the post-natal organism.

Dentinal repair in the post-natal organism occurs through the activity of specialized cells, odontoblasts, that are thought to be maintained by an as yet undefined precursor population associated with pulp tissue (1). To date, the identification and

isolation of an odontogenic progenitor population from adult dental pulp tissue has never been performed. It is known that in certain conditions, cultures of pulp cells derived from early developing dental root tissue and pulp tissue can develop an odontoblast-like appearance with the capacity to form mineralized nodules *in vitro* (4) a trait normally attributed to cultures of bone or bone marrow cells (5, 6). More is known about the characteristics of multipotent bone marrow stromal cells (BMSCs) and their potential to develop into osteoblasts, chondrocytes, adipocytes, myelosupportive fibrous-stroma, and perhaps even muscle and neural tissues (7-12). They are characterized by their high proliferative capacity *ex vivo*, while maintaining their ability to differentiate into multiple stromal cell lineages.

Because the prior art does not provide for regenerating dentin/pulp tissue or producing a human tooth for restorative purposes, there exists a great need to find a means for producing tissue that can differentiate into a functional tooth. In this study, a clonogenic, rapidly proliferative population of cells from adult human dental pulp was isolated. These dental pulp stem cells (DPSCs) were then compared to human bone marrow stromal cells (BMSCs), known precursors of osteoblasts. Although they share a similar immunophenotype *in vitro*, functional studies showed that DPSCs produced only sporadic, but densely calcified nodules, and did not form adipocytes, whereas BMSCs routinely calcified throughout the adherent cell layer with clusters of lipid-laden adipocytes. When DPSCs were transplanted into immunocompromised mice, they generated a dentin-like structure lined with human odontoblast-like cells that surrounded a pulp-like interstitial tissue. In contrast, BMSCs formed lamellar bone containing osteocytes and surface lining osteoblasts, surrounding a fibrous vascular tissue with active hematopoiesis and adipocytes. This study is the first to isolate post-natal human dental pulp stem cells that have the ability to form a dentin/pulp complex.

The present invention overcomes the previous limitations and shortcomings in the art by providing a human adult dental pulp stem cell (DPSC) that can differentiate into dentin/pulp tissue and a method for regenerating dentin/pulp and for producing a human tooth.

SUMMARY OF THE INVENTION

The present invention provides a culture of isolated adult human dental pulp stem cells.

The present invention also provides a method of regenerating human dentin/pulp tissue, comprising a) contacting a cell from a culture of isolated adult human dental pulp stem cells with hydroxyapatite/tricalcium phosphate and b) transplanting the cell from step a) into a mammal.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1E. Colony forming efficiency and cell proliferation *in vitro*. Representative high (A) and low (B) density colonies after 14 days. The morphology is typical of fibroblast-like cells (C). The incidence of colony forming cells from dental-pulp tissue and bone marrow at various plating densities indicates that there are more clonogenic cells in dental pulp than in bone marrow (D). The number of BrdU positive cells was expressed as a percentage of the total number of cells counted for DPSCs and BMSCs (E). Statistical significance (*) was determined using the student t-test ($p \geq 0.05$).

Figures 2A-2L. Immunophenotype of cultured DPSCs. Studies based on immunoperoxidase reactivity were performed on first passage cultures of DPSCs. Representative staining patterns are shown for: integrin β1, (A); CD44, (B); collagen type I, (C); collagen type III, (D); FGF-2, (E); osteonectin, (F); osteocalcin, (G); MUC-18, (H); α-smooth muscle actin, (I); osteopontin, (J); VCAM-1, (K). Endogenous, alkaline phosphatase activity is shown in (L).

Figures 3A-3D. Developmental potential *in vitro*. Adherent layers of cultured DPSCs (A & B), and BMSCs (C & D), are shown with Alizarin Red staining as a measure of calcium accumulation following 6 weeks of induction with L-ascorbate-2-phosphate and dexamethasone with inorganic phosphate (A & C). After 6 weeks in the same medium but without inorganic phosphate, lipid accumulation was noted in BMSCs (D), but not in DPSCs (B).

Figures 4A-4F. Developmental potential *in vivo*. Cross sections are representative of DPSC transplants, (A, C, D) and BMSC transplants, (B, E, F) 6 weeks post-transplantation and stained with hematoxylin and eosin. In the DPSC transplants, the HA/TCP carrier surfaces (c) are lined with a dentin-like matrix (d), surrounding a pulp-like tissue with blood vessels (bv) and an interface layer of odontoblast-like cells (od) (A). A magnified view of the dentin matrix (d) highlights the odontoblast-like layer (od) and odontoblast processes (arrow) (C). Polarized light demonstrates perpendicular alignment (dashed lines) of the collagen fibers to the forming surface (D). In BMSC transplants, lamellar bone (b) is formed on the HA/TCP surfaces (c) and surrounds a vascular, hematopoietic marrow organ (hp) with accumulated adipocytes (a) (B). A magnified view shows that the new bone contains osteocytes (oc), embedded within the calcified matrix, and osteoblasts (ob) lining the bone surfaces (E). With polarized light, collagen fibrils are seen to be deposited parallel with the forming surface (dashed lines) (F).

Figures 5A-5B. In situ hybridization for the human specific alu DNA sequence. Alu positive cells in the pulp tissue (large arrow) and odontoblast-like layer (small arrow) adjacent to the dentin matrix (d) are easily recognized in 6 week DPSC transplants (A). Osteocytes encased in the new bone matrix (small arrow) and the osteoblasts lining the bone (b) surfaces (large arrow) show positive reactivity with the alu probe in the BMSC transplants (B). Hematopoietic elements (hp) in the marrow-like organ fail to show reactivity with the alu probe.

Figures 6A-6C. Expression of the human specific Dentin Sialophosphoprotein (DSPP), osteocalcin (OC), bone sialoprotein (BSP) mRNA in DPSC transplants. Transcripts for DSPP, BSP, OC and GAPDH were detected by RT-PCR using total RNA isolated from 6 week old DSPC transplants (A). DSPP positive cells were also found in the pulp tissue and odontoblast layer (arrow) adjacent to the dentin matrix (d) by in situ hybridization (B). Specificity of the probe was verified by hybridization in the odontoblast layer (arrow) of human dental pulp (p) tissue (C). No reactivity of the DSPP specific probe was detected in human bone, bone marrow and muscle tissue.

Figures 7A-7D. Immunohistochemical staining of human DSPP. Bone and associated marrow elements were negative for DSPP antibody staining in BMSC transplants (A). Newly formed dentin was positive for DSPP antibody staining in DPSC transplants (arrow, B). Normal human dentin and odontoblasts in human pulp were positive for DSPP (C and D, respectively).

Figures 8A-8B. Dentin formation in DPSC transplants. Trichrome (blue) staining of dentin (arrow) in eight week old human DPSC transplants (A). Transverse section of a normal tooth depicting trichrome staining (arrow) of dentin in situ (B).

DETAILED DESCRIPTION OF THE INVENTION

As used herein, "a," "an" or "the" may mean one or more. For example, "a" cell may mean one or more cells. Moreover, "the" cell may mean one or more than one cell.

The present invention provides a culture of isolated adult human dental pulp stem cells. Adult human dental pulp stem cells can be isolated from any human permanent tooth. Alternatively, the dental pulp stem cells of the invention can be isolated from a human subject at least about 18 years of age. "Isolated" as used herein means the cell of this invention is sufficiently free of contaminants or other cell types with which the cell of the present invention is naturally found. Moreover, the isolated cell of the present invention is present in such concentration as to be the only significant cell in the sample. "Isolated" does not require that the preparation be technically pure (homogeneous), but it is sufficiently pure to provide the cell in a form in which it can be used therapeutically or for research.

A "stem" cell is a cell that is multipotential, clonogenic, highly proliferative and capable of regenerating tissue. Thus, a stem cell has the ability to either influence other cells in its microenvironment to differentiate into a specific cell line or can itself differentiate into another cell type that has a specific function. Thus, an adult human dental pulp stem cell of the present invention is a cell obtained from the pulp tissue of a tooth from an adult human being. The stem cell of the present invention can differentiate into a more mature and functional cell, for example an odontoblast. An odontoblast is a cell found in the oral cavity of a mammal that produces dentin, a tissue that comprises and envelops a fibrovascular pulp and is covered by an outer enamel layer of a fully-formed tooth. The dental pulp stem cell of the present invention can

produce a dentin/pulp tissue structure *in vivo* and *in vitro*. This structure is exemplified in Figure 1.

The present invention provides a stem cell that is not adipogenic. Thus, the DPSC of the present invention does not differentiate into a fat cell. Moreover, the stem cell of the present invention does not produce bone sialoprotein. Thus, the stem cell of the present invention is not a bone marrow stem cell, a cell which can differentiate into a fat cell and produces bone sialoprotein. However, the stem cell of the present invention does produce odontoblast-specific dentin sialoprotein and dentin phosphoprotein, each protein encoded by the odontoblast-specific gene known as dentin sialophosphoprotein (DSPP).

The present invention provides a method of regenerating human dentin/pulp tissue, comprising a) contacting a cell from a culture of isolated adult human DPSCs with a mineral source and b) transplanting the cell from step a) into a mammal. The stem cell of the present method is mixed with a mineral source, for example, hydroxyapatite/tricalcium phosphate. This cell/mineral composition can then be transplanted into a mammal by methods known to a person of skill in the art. The stem cell/mineral composition can be transplanted into an immunocompromised mammal, for example a mouse, wherein the transplanted tissue can produce human dentin/pulp tissue that can be harvested for transplantation into a human being. The mammal may be a mouse, sheep, cow or any mammal used for transplantation studies and known to a person of skill in the art, including a human. Further, a human recipient of the stem cell/mineral composition can be an intermediate host from whom the dentin/pulp tissue can be harvested for further transplantation into another human dental patient. Alternatively, the human recipient of the stem cell/mineral composition can be both the donor and only recipient of the transplanted tissue.

Moreover, the present invention provides a method of producing human dentin/pulp tissue, comprising a) contacting a cell from a culture of isolated adult human DPSCs with a mineral source and b) transplanting the cell from step a) into a mammal. The stem cell of the present invention can be mixed with a mineral source, for example hydroxyapatite/tricalcium phosphate.

A person of skill in the art can transplant the stem cell/mineral composition into various sites of a mammal including subcutaneous tissue and oral tissue. It is contemplated that a person of skill in the art can transplant the stem cell/mineral composition into a tooth socket and, thereby, produce a dentin/pulp tissue in a human patient.

Moreover, the transplanted stem cell/mineral composition can be transplanted with or contacted *in vitro* with ameloblasts, cells that produce enamel, to produce a fully-formed functional tooth, comprising dentin/pulp tissue covered by enamel. Similarly, artificial crowns made from bio-compatible materials that mimic the hard enamel layer of teeth can be produced and used as a template. The transplanted stem cell/mineral composition can be transplanted directly into the cavity of an artificial crown where the cells can differentiate into dentin/pulp tissue *in vivo*, thereby forming a functional tooth.

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compositions and/or methods claimed herein are made and evaluated, and are intended to be purely exemplary of the invention and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.), but some errors and deviations should be accounted for. The present invention is more particularly described in the following

examples which are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

EXAMPLES

Subjects and cell culture.

Normal human impacted third molars were collected from adults (19-29 years of age) at the Dental Clinic of the National Institute of Dental & Craniofacial Research under approved guidelines set by the NIH Office of Human Subjects Research. Tooth surfaces were cleaned and cut around the cementum-enamel junction using sterilized dental fissure burs to reveal the pulp chamber. The pulp tissue was gently separated from the crown and root and then digested in a solution of 3 mg/ml collagenase type I (Worthington Biochem, Freehold, NJ) and 4 mg/ml dispase (Boehringer Mannheim, GMBH, Germany) for one hour at 37°C. Single cell suspensions were obtained by passing the cells through a 70 μm strainer (Falcon, BD Labware, Franklin Lakes, NJ). Bone marrow cells, processed from marrow aspirates of normal human adult volunteers (20-35 years of age), were purchased from Poietic Technologies, Gaithersburg, Maryland, and then washed in growth medium. Single cell suspensions (0.01 to 1 x 10^5/well) of dental pulp and bone marrow were seeded into 6-well plates (Costar, Cambridge, MA) with alpha Modification of Eagle's Medium (GIBCO BRL, Grand Island, NY) supplemented with 20% fetal calf serum (Equitech-Bio Inc, Kerrville, TX), 100 μM L-ascorbic acid 2-phosphate (WAKO, Tokyo, Japan), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (Biofluids Inc, Rockville, MD), then incubated at 37°C in 5% CO_2. To assess colony-forming efficiency, day 14 cultures were fixed with 4% formalin, and then stained with 0.1% toluidine blue. Aggregates of ≥50 cells were scored as colonies. Conditions for the induction of calcified bone matrix deposition *in vitro* were as reported previously (6). The proliferation rate of

sub-confluent cultures (first passage) of DPSCs and BMSCs was assessed by bromodeoxyuridine (BrdU) incorporation for 24 hours, using a Zymed Laboratories BrdU staining Kit (Vector Laboratories, Burlingame CA).

Immunohistochemistry.

Primary DPSCs and BMSCs were sub-cultured into 8-chamber slides (2 x 10^4 cells/well) (NUNC Inc, Naperville, IL). The cells were fixed in 4% formalin, and then reacted with saturating levels of primary antibodies and the corresponding control antibodies using a Zymed broad spectrum immunoperoxidase kit (Vector Laboratories). Antibodies used were: Mouse (IgG) control (Caltag, Burlingame, CA). Rabbit (Ig) control, TUK4 (anti-CD14), QBEND 10 (anti-CD34), 2B11/PD7 (anti-CD45), M318 (anti-MyoD), 1A4 (anti-α smooth muscle actin), 2F11 (anti-neurofilament), (Dako, Carpinteria, CA); H9H11 (anti-CD44), 6G10 (anti-VCAM-1) (Dr. P.J. Simmons, HCCR, Adelaide, South Australia); CC9 (anti-MUC-18) (Dr. S. Gronthos NIDCR/ NIH, MD); MAB1343 (anti-COL III), MAB1959 (anti-β1) (Chemicon, Temecula, CA); LF67 (anti-COL I), LF32 (anti-OC), BON-1 (anti-ON), LF100 (anti-BSP), LF123 (anti- OP) (Dr. L. Fisher, NIDCR/ NIH, MD); MAB1104 (anti-COL II) (RDI, Flanders, NJ); E-8 (anti-PPARγ), 147 (anti-FGF-2), (Santa Cruz, Santa Cruz, CA). Working dilutions of rabbit serum (1/500), monoclonal supernatants (1/4) and purified antibodies (10 μg/ml) were used.

Histochemistry.

Secondary DPSC and BMSC cultures were washed in PBS and then fixed with 4% formalin. Alkaline phosphatase activity was assessed using a Sigma *in vitro* alkaline phosphatase substrate kit (85L-2). Calcium deposits were detected by treatment with 2% Alizarin Red S (pH 4.2).

Transplantation.

Approximately 5.0 x10^6 of DPSCs and BMSCs (third passage) were mixed with 40 mg of hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic powder (Zimmer Inc, Warsaw, IN) and then transplanted subcutaneously into the dorsal surface of 10-week-old immunocompromised beige mice (NIH-bg-nu-xid, Harlan Sprague Dawley, Indianapolis, IN) as previously described (9). These procedures were performed in accordance to specifications of an approved small animal protocol (NIDCR #97-024). The transplants were recovered at 6 weeks post-transplantation, fixed with 4% formalin, decalcified with buffered 10% EDTA (pH 8.0), and then embedding in paraffin. Sections (5 m) were deparaffinized and stained with H&E.

RT-PCR.

Total RNA was prepared from collagenase/dispase digested cell suspensions of six week old DPSC transplants, using RNA STAT-60 (TEL-TEST Inc. Friendswood, TX). First-strand cDNA synthesis was performed using a first-strand cDNA synthesis kit (GIBCO BRL, Life Technologies) using an oligo-dT primer. First strand cDNA (2 µl) was diluted in a 50 µl PCR reaction of 1X PCR reaction buffer: 1.5 mM $MgCl_2$, 200 mM each dNTP, 0.2 units of AmpliTaq DNA Polymerase (Perkin-Elmer Inc, Norwalk, CT) and 10 pMol of each human specific primer sets: BSP (sense (SEQ ID NO.:1) 5'-CTATGGAGAGGACGCCACGCCTGG-3' (S(antisense, (SEQ ID NO.:2) 5'- CATAGCCATCGTAGCCTTGTCCT-3'), OC (sense, (SEQ ID NO.:3) 5'-CATGAGAGCCCTCACA-3'; antisense, (SEQ ID NO.:4) 5'-AGAGCGACACCCTAGAC-3'), DSPP (sense (SEQ ID NO.:5) 5'-GGCAGTGACTCAAAAGGAGC-3'; antisense, 5'-(SEQ ID NO.:6) TGCTGTCACTGTCACTGCTG-3'), GAPDH (sense, (SEQ ID NO.:7) 5'-AGCCGCATCTTCTTTTGCGTC-3'; antisense (SEQ ID NO.:8) 5'-TCATATTTGGCAGGTTTTTCT-3'). The reactions were incubated in a PCR Express Hybaid thermal cycler (Hybaid, Franklin, MA) at 94°C for 2 minutes for 1 cycle then

94°C/(45 sec), 56°C/(45 sec), 72°C/(60 sec) for 35 cycles, with a final 10 minute extension at 72°C. Following amplification, 10 µl of each reaction was analyzed by 1.5% agarose gel electrophoresis, and visualized by ethidium bromide staining.

In situ hybridization.

A digoxigenin-labeled probe for human specific alu repetitive sequence was prepared by PCR using primers for alu as previously described (13). Similarly, a digoxigenin-labeled probe specific for human DSPP mRNA was also prepared using the DSPP primers under the same PCR conditions as described above. The specificity of both probes was verified by DNA sequencing. Unstained sections were deparaffinized and hybridized with either the digoxigenin-labeled alu probe (9) or the DSPP probe using the mRNAlocator-Hyb Kit (Cat # 1800; Ambion, Inc., Austin TX). After hybridization, the presence of both alu and DSPP mRNA in tissue sections was detected by immunoreactivity with an anti-digoxigenin alkaline phosphatase conjugated Fab fragments (Boehringer Mannheim).

Isolation of clonogenic populations of DPSCs.

Osteoprogenitors can be isolated from aspirates of bone marrow by their ability to adhere to a plastic substratum, and with appropriate stimulation, begin to proliferate (13-15). Each colony originates from a single progenitor cell (colony forming unit-fibroblast, CFU-F) and displays a wide variation in cell morphology and growth potential (13-18). Herein, the presence of a clonogenic cell population in dental pulp tissue (Fig. 1A and B) is demonstrated for the first time. The cells within each colony were characterized by a typical fibroblast-like morphology (Fig. 1C) analogous to the progeny of human bone marrow CFU-F (19). The frequency of colony forming cells derived from dental pulp tissue (range 22 to 70 colonies per 10^4 cells plated) was significantly higher in comparison to the incidence of bone marrow CFU-F (range 2.4 to 3.1 colonies/ 10^4 cells plated) over similar plating densities (0.1 to 2.5 x 10^4 cells

plated) (Fig. 1D). In addition, the number of proliferating cells in DPSC cultures was also significantly higher (mean 72% BrdU positive cells ± 3.48 SEM, n=3) when compared to BMSC cultures (46% BrdU positive cells ± 1.96 SEM, n=3) using the BrdU uptake method (t-test $p \leq 0.05$) (Fig. 1E).

Characterization of the immunophenotype of DPSCs *in vitro*.

Immunohistochemical studies were performed to characterize the progeny of the DPSC and BMSC clonogenic populations, using a large panel of antibodies specific to known antigens associated with different phenotypes. Typical immunoreactivity profiles for both cell populations are shown in Table 1. Primary cultures of DPSC and BMSC failed to react with the hematopoietic markers CD14 (monocyte/ macrophage), CD45 (common leukocyte antigen), CD34 (hematopoietic stem/progenitor cells/endothelium) and other markers such as MyoD (smooth muscle), neurofilament (nerve), collagen type II (cartilage) and PPARγ (fat). In general, DPSCs and BMSCs exhibited a similar expression pattern for a variety of markers associated with endothelium (VCAM-1 and MUC-18), smooth muscle (α-smooth muscle actin), bone (alkaline phosphatase, type I collagen, osteonectin, osteopontin, osteocalcin) and fibroblasts (type III collagen, FGF-2). The bone matrix protein, bone sialoprotein, was absent in DPSC cultures, but present at low levels in BMSC cultures. Representative immunoreactivity patterns for DPSC are shown in (Fig. 2). Many of the markers were not uniformly expressed, but found in subsets of cells, indicating that the DSPC population is heterogeneous, as has been shown for the BMSC population.

Differentiation potential of DPSCs *in vitro*.

Long-term cultures (5 to 6 weeks) of DPSCs grown in the presence of L-ascorbate-2-phosphate, the glucocorticoid, dexamethasone, and inorganic phosphate demonstrated the capacity to form Alizirin Red positive condensed, nodules with high levels of calcium (Fig. 3A). The deposits were sparsely scattered throughout the

adherent layer as single mineralized zones. In contrast, BMSC cultures produced extensive sheets of calcified deposits over the entire adherent layer following 3 to 4 weeks of induction (Fig. 3C). After 6 weeks of stimulation with dexamethasone, there was no evidence of adipogenesis in primary DPSC cultures (Fig. 3C) where as clusters of lipid-containing adipocytes were detected in primary cultures of BMSC as early as two weeks (Fig. 3D).

Ex vivo expanded DPSCs can generate a dentin/pulp structure *in vivo*. Because complete developmental potential and formation of an appropriate histological structure often can not be fully realized *in vitro*, DPSCs were transplanted in conjunction with hydroxyapatite/tricalcium phosphate (HA/TCP) powder into immunocompromised mice. After 6 weeks post-transplantation, DPSCs generated a dentin-like structure lining the surfaces of the HA/TCP particles, comprised of a highly ordered collagenous matrix deposited perpendicular to the odontoblast-like layer when viewed by polarized light (Fig. 4A, C, D). Immunological studies demonstrated that the matrix was predominantly composed of collagen type I. The odontoblast-like cells extended cytoplasmic processes into the dentinal matrix, which interfaced with a pulp-like interstitial tissue infiltrated with blood vessels. The pulp and odontoblast-like cells were found to be of donor origin based on their reactivity to the human alu specific probe (Fig. 5A). Furthermore, the DPSC transplants expressed human specific transcripts for dentin matrix components, including bone sialoprotein, osteocalcin and dentin sialophosphoprotein (DSPP) by RT-PCR and in situ hybridization (Fig. 6). The corresponding BMSC transplants formed distinct lamellae of bone on the surface of the HA/TCP where the collagen fibers were aligned parallel to the osteoblasts on the bone forming surfaces (Fig. 4B, E, F). Osteocytes, entombed within the bone matrix, and osteoblasts were also found to be of donor origin (Fig. 5B). Newly formed bone surrounded an interstitial tissue that was infiltrated by a sinusoid network resembling a marrow-like organ, with extensive areas of active hematopoiesis and adipocyte

accumulation. Interestingly, the DPSC transplants failed to support any hematopoiesis or to initiate adipocyte formation even 4 months post transplantation.

Immunohistochemical staining for human DSPP.

Transplantation of DPSCs and BMSCs was performed as described above. The transplants were recovered 8 weeks post-transplantation and fixed for 24 hours with 4% freshly prepared paraformadehyde, and decalcified for 7 days with 10% EDTA (pH 8.0). The transplants were then transferred to 70% ethanol and embedded into paraffin block. The DSPP antibody was from Dr. Larry Fisher (NIDCR/NIH). After deparaffinization, rehydration, and elimination of endogenous peroxidase, the sections were incubated with the DSPP primary antibody at room temperature for 1 hour. Then Histostain SP Kits were used for second antibodies and enzyme conjugate incubation according to the instruction (Zymed Laboratories Inc. South San Francisco, CA, USA). Human pulp and demineralized dentin tissues were used as positive controls. This procedure showed that newly formed dentin in DPSCs transplants is positive for dentin specific DSPP antibody (Fig. 7). This is additional evidence that human DPSCs differentiate into odontoblasts to regenerate human dentin *in vivo*.

Trichrome staining of dentin formation in DPSC transplants.

Transplantation of DPSCs and BMSCs was performed as described above. Eight week old human DPSC transplants were fixed for 24 hours with 4% freshly prepared paraformaldehyde, and transferred into 70% ethanol and plastic embedded. The Trichrome staining (GOMORI) kit (Sigma, #HT10516) was used for the staining according to the manufacturer's protocol. Normal tooth plastic-embedded sections were used as positive controls. This staining showed that DPSCs regenerate dentin comprising similar mineralized collagen structure as normal human dentin (Fig. 8).

The data presented here demonstrate for the first time that post-natal dental pulp contains cells that are clonogenic, highly proliferative and capable of regenerating a tissue, properties that effectively define them as stem cells. Although muscle, nervous tissue, and dentin-associated tissue do not remodel during post-natal life, they all contain stem cells that have the ability to differentiate in response to injury. The transplantation of human DPSCs into immunocompromised mice provides a new model by which to further characterize these stem cells. Furthermore, the amount of dentin and pulp-like tissue formed in these transplants far exceeds the amount that would be generated in situ during the lifetime of an organism. Consequently, the present isolation of a large number of DPSCs from a single tooth allows for dentinal repair of a number of teeth. Further, through the use of carriers with appropriate shape and composition in conjunction with *ex vivo* expanded DPSCs, the fabrication of a viable dental implant is provided.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

It will be apparent to those skilled in the art that various modifications and variations can be made in the present invention without departing from the scope or spirit of the invention. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

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Table 1. Immunohistochemical analysis of human DPSCs and BMSCs *in vitro*.

Marker	DPSC-1	DPSC-2	BMSC
CD14	-	-	-
CD34	-	-	-
CD44	++	++	++
CD45	-	-	-
Integrin β1	++/+	++/+	++
VCAM-1	+	+	++
MyoD	-	-	-
α-SM actin	++/-	++/-	++/+/-
Neurofilam.	-	-	-
MUC-18	++/-	++/+/-	++/+/-
Collagen-I	+	++	++/+
Collagen-II	-	-	-
Collagen-III	++/+	++/+	++/+
Osteocalcin	++/+	++/+	+/-
Osteonectin	++/+	++	++/+
BSP	-	-	+/-
Osteopontin	+/-	+/-	+/-
Alk Phos	++/+/-	++/+/-	++/+/-
PPARγ	-	-	-
FGF-2	++/+	++	++/+

(++) Strong staining, (+) Weak staining,

(-) Negative, (/) Subpopulation

What is claimed is:

1. A culture of isolated adult human dental pulp stem cells.

2. The culture of claim 1, wherein the stem cells can differentiate into odontoblasts.

3. The culture of claim 1, wherein the stem cells can regenerate a dentin/pulp structure *in vivo*.

4. The culture of claim 1, wherein the stem cells are not adipogenic.

5. The culture of claim 1, wherein the cells do not produce bone sialoprotein.

6. A method of regenerating human dentin/pulp tissue, comprising:

a) contacting a cell from the culture of claim 1 with a mineral source; and

b) transplanting the cell from step a) into a mammal.

7. The method of claim 6, wherein the mineral source is hydroxyapatite/tricalcium phosphate.

8. A method of producing human dentin/pulp tissue, comprising:

a) contacting a cell from the culture of claim 1 with a mineral source; and

b) transplanting the cell from step a) into a mammal.

9. The method of claim 8, wherein the mineral source is hydroxyapatite/tricalcium phosphate.

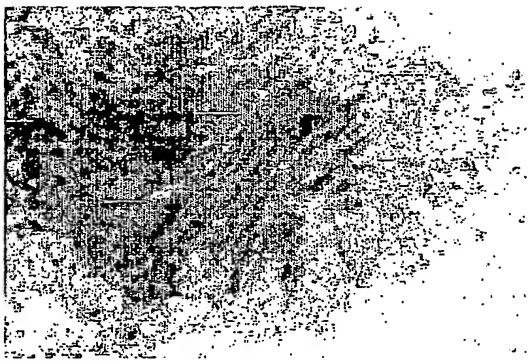


FIG. 1A

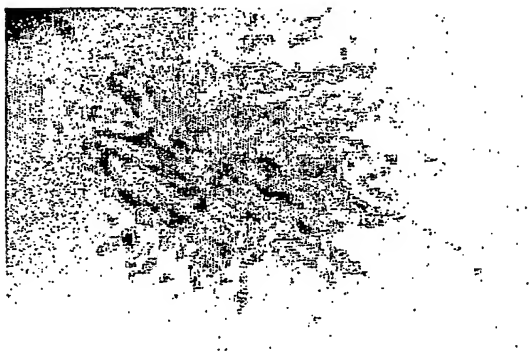


FIG. 1B

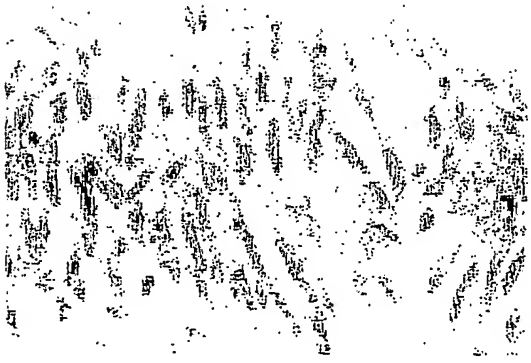


FIG. 1C

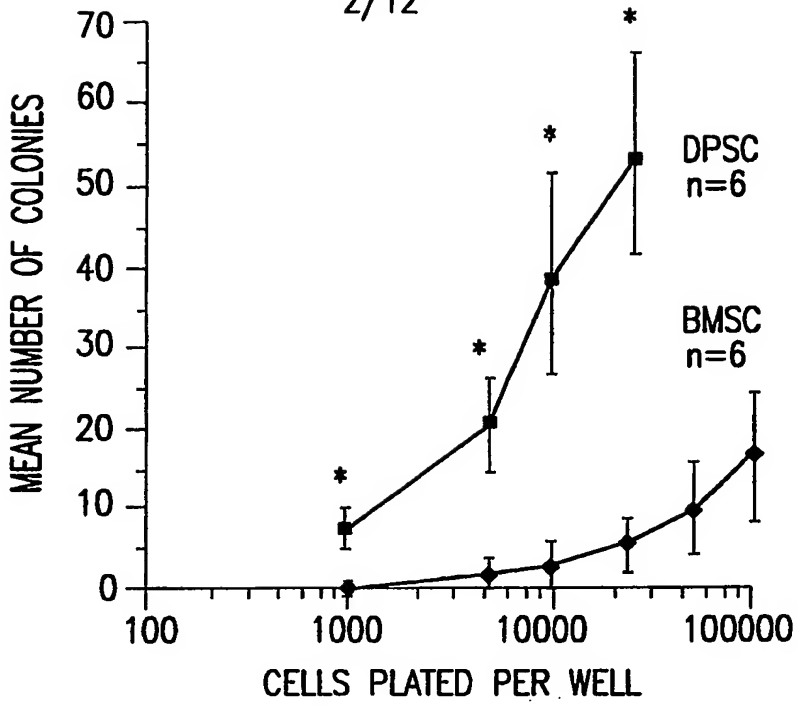

MEAN NUMBER OF COLONIES
70
60
50
40
30
20
10
0
100
1000
10000
100000
CELLS PLATED PER WELL
DPSC
n=6
BMSC
n=6
*

FIG.1D

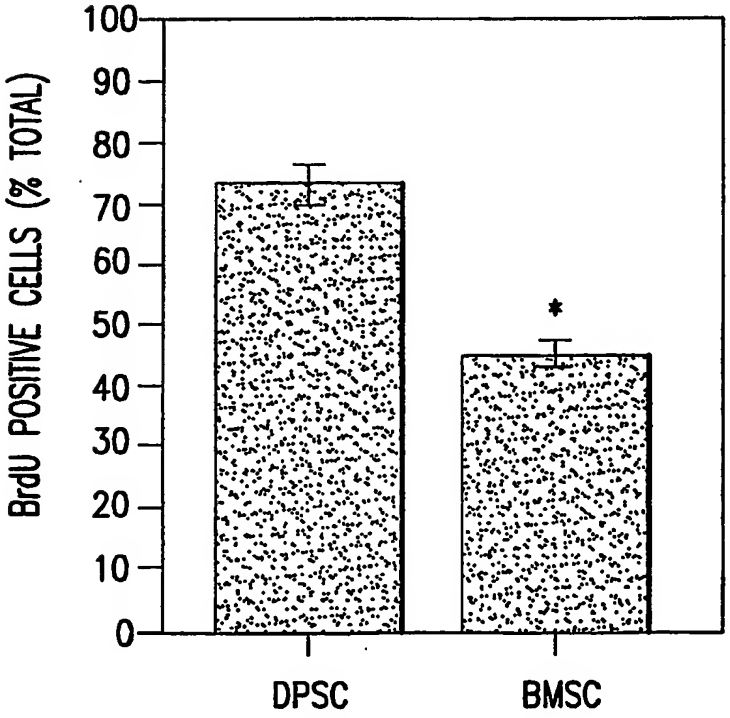

BrdU POSITIVE CELLS (% TOTAL)
100
90
80
70
60
50
40
30
20
10
0
DPSC
BMSC
*

FIG.1E

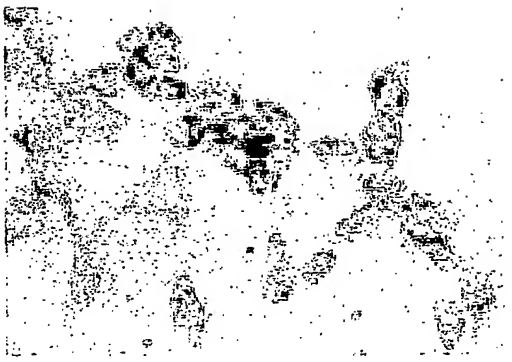


FIG.2A

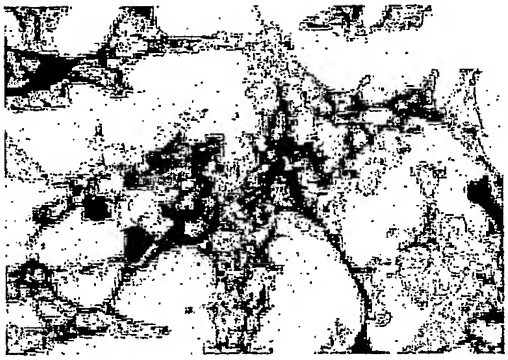


FIG.2B

FIG.2C

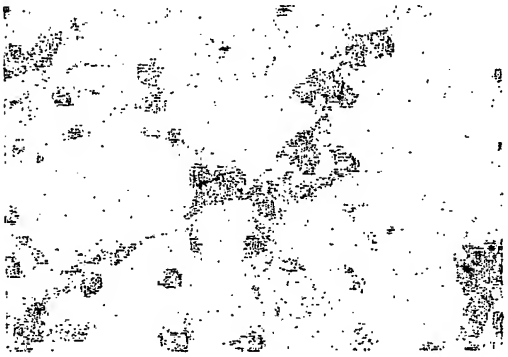


FIG.2D

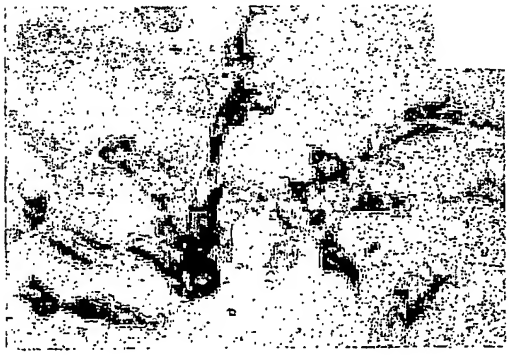


FIG.2E

FIG.2F

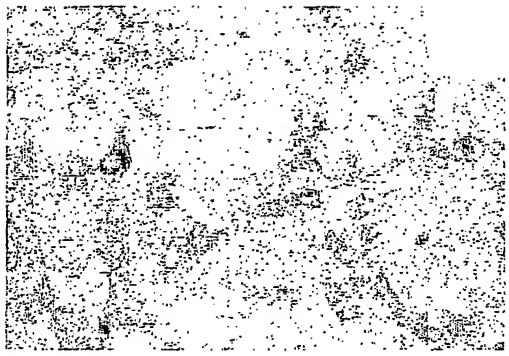


FIG.2G

FIG.2H

FIG.2I

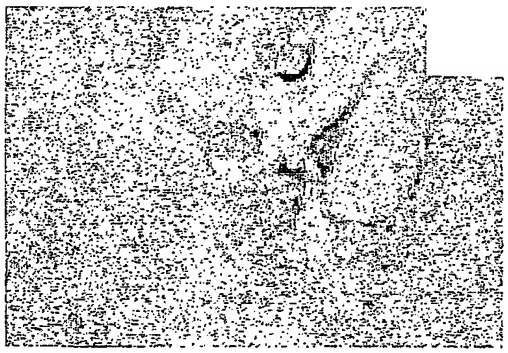


FIG.2J

FIG.2K

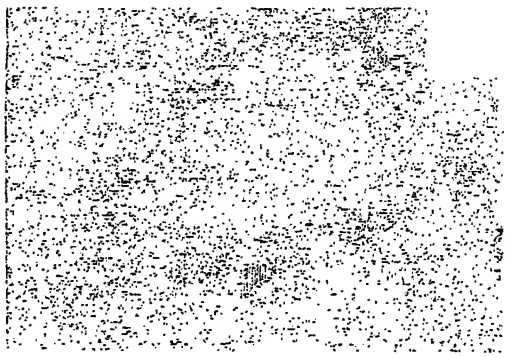


FIG.2L

FIG.3A

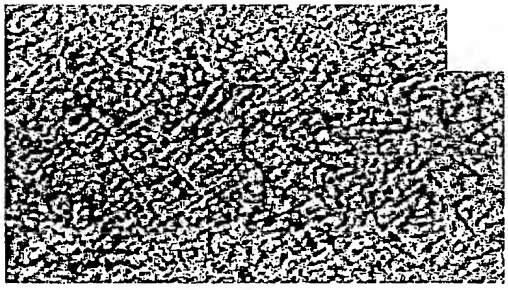


FIG.3B

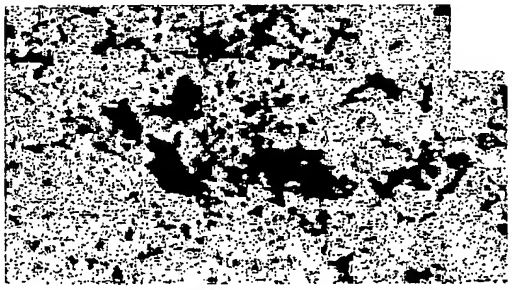


FIG.3C

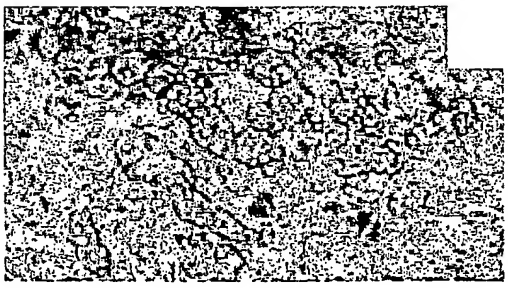


FIG.3D

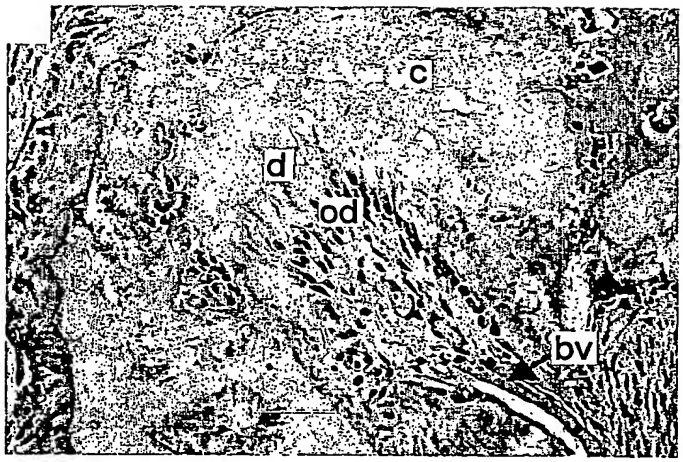


FIG.4A

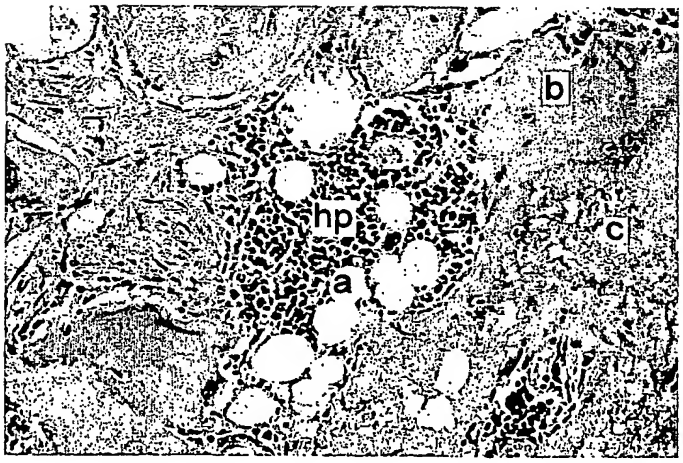


FIG.4B

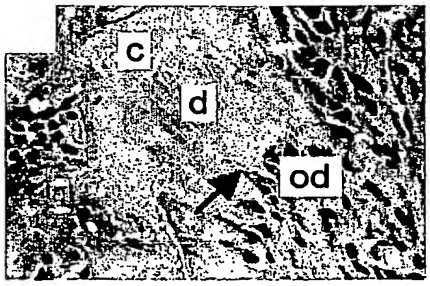


FIG.4C

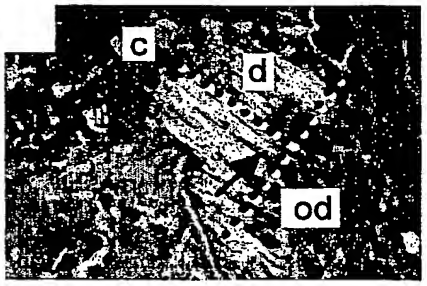


FIG.4D

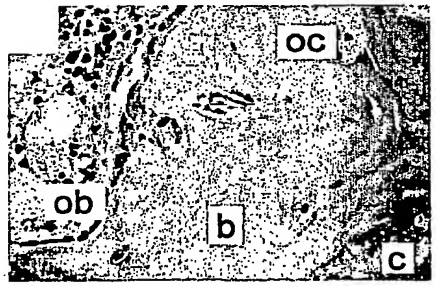


FIG.4E

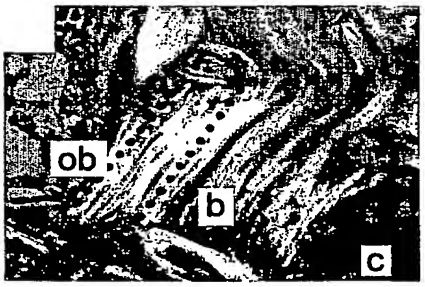


FIG.4F

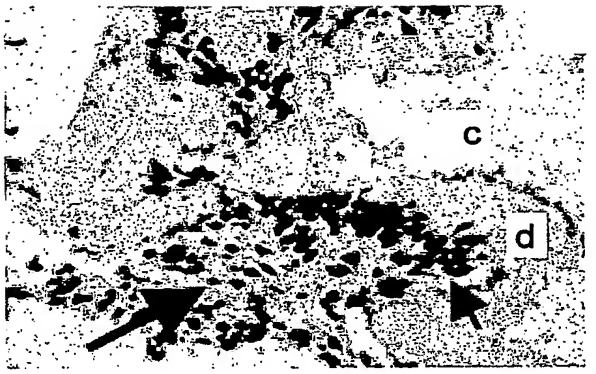


FIG.5A

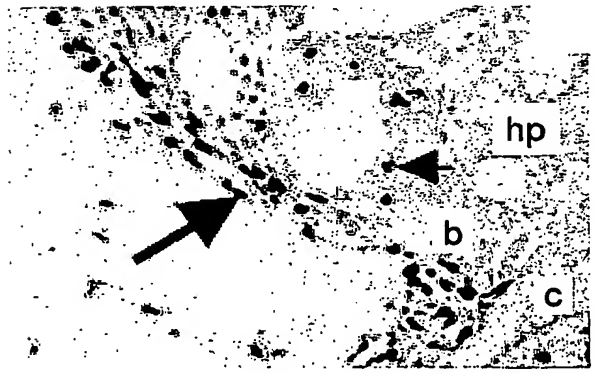


FIG.5B

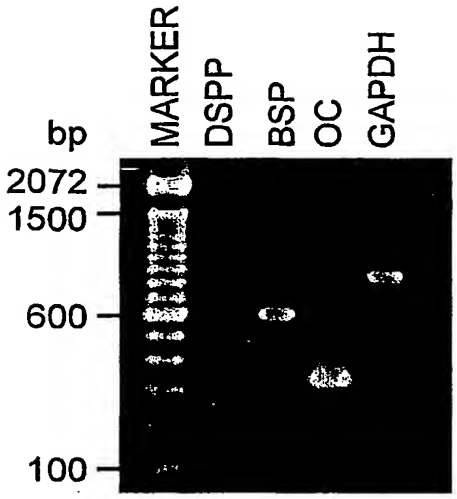
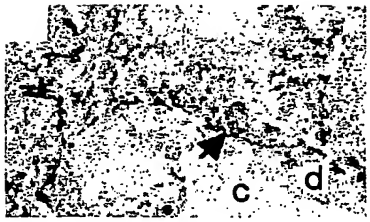


FIG.6A



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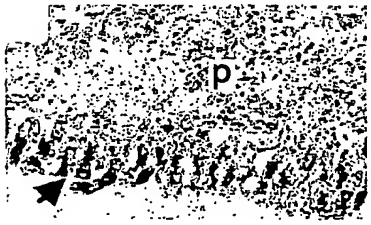

FIG.6C

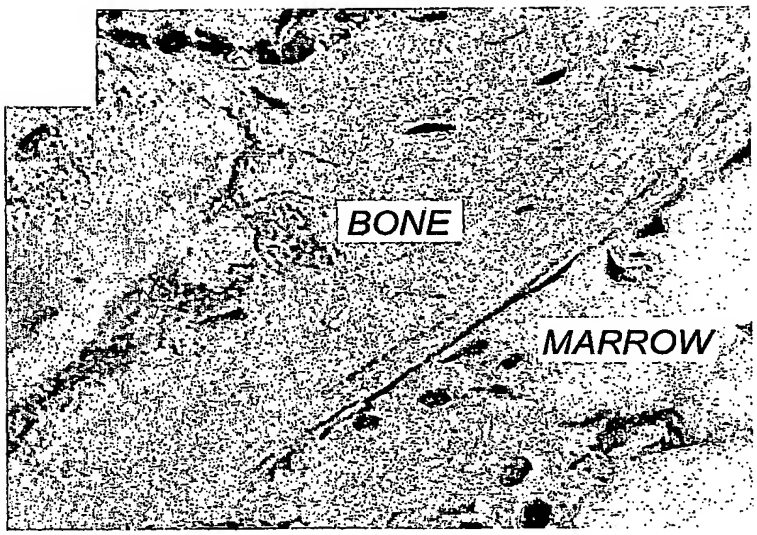

FIG.7A

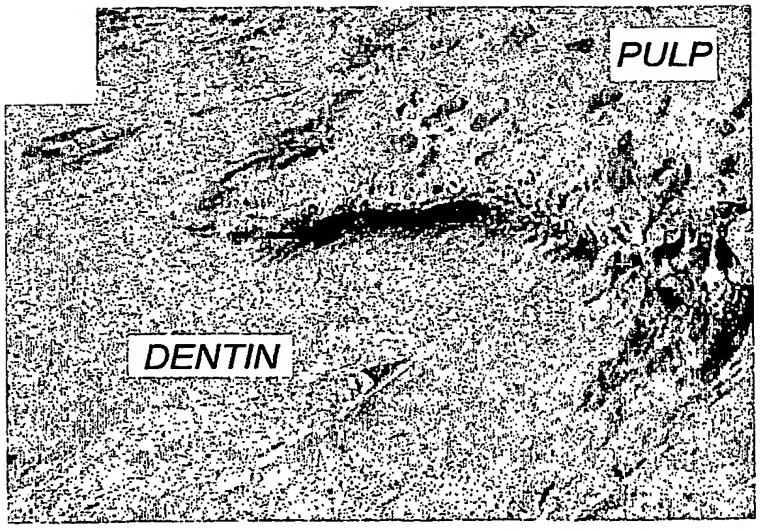

FIG.7B

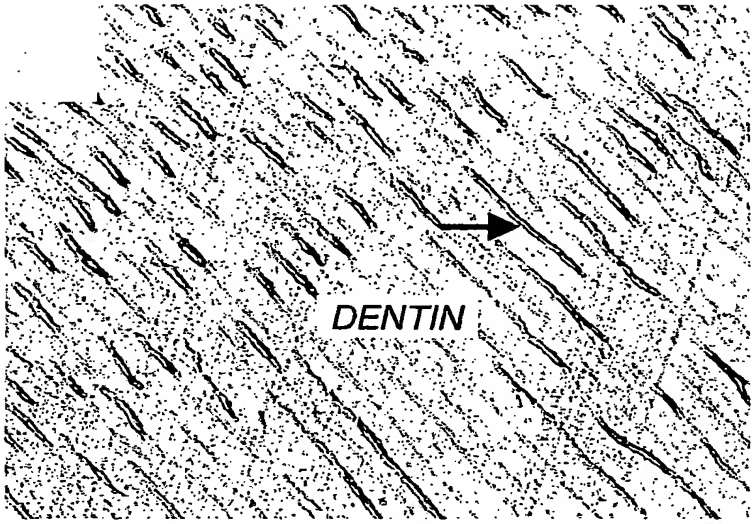

FIG.7C

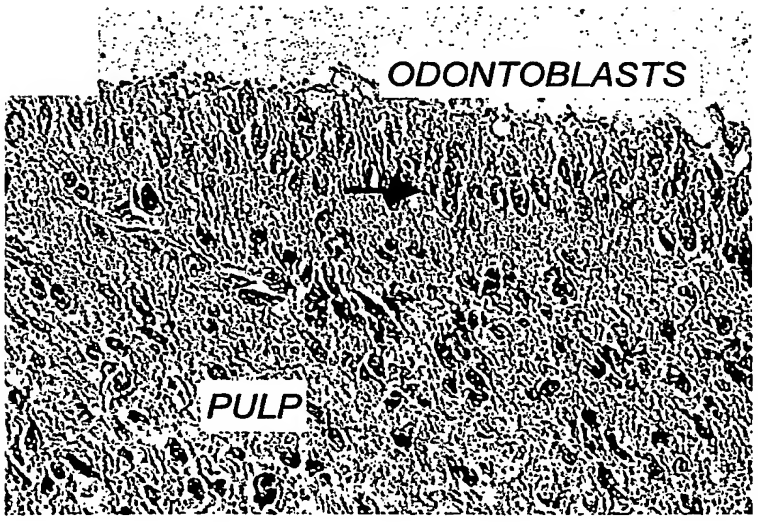

FIG.7D

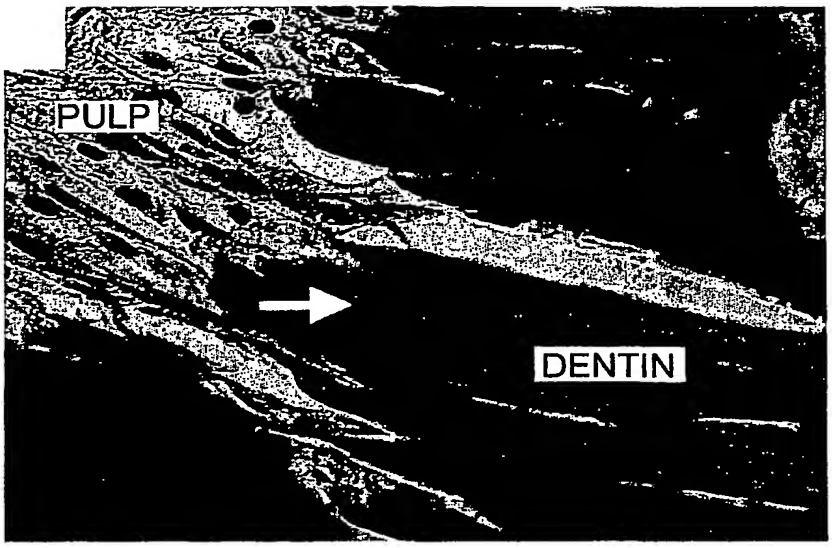

FIG.8A

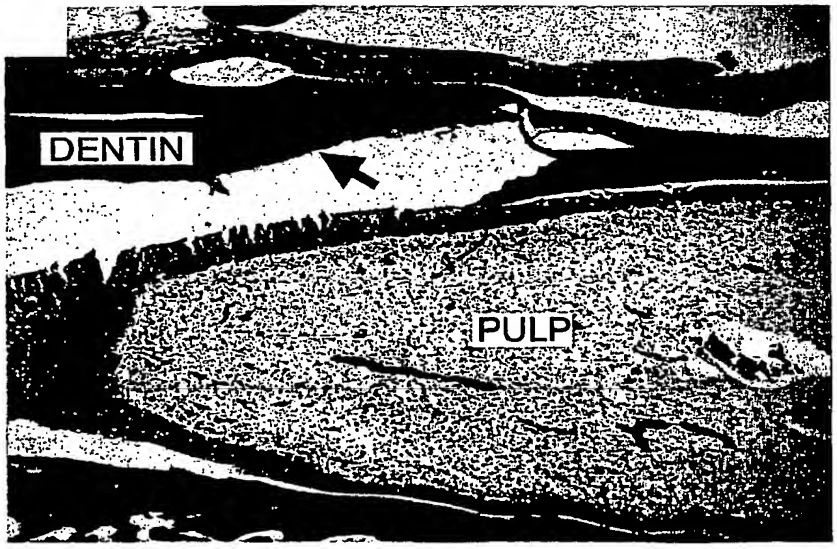

FIG.8B

SEQUENCE LISTING

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Stan Gronthos
Pamela Gehron Robey

<120> ADULT HUMAN DENTAL PULP STEM CELLS *IN VITRO* AND *IN VIVO*

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<150> 60/219,989
<151> 2000-07-21

<160> 8

<170> FastSEQ for Windows Version 4.0

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<223> Description of Artificial Sequence; Note =
synthetic construct

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